Table II. Simultaneous optimization of framework and CDR residues.

Library	Clone	Kd (nM)	Murine Fr Residues*	CDR Mutations
	chimeric	48.3	(43)	0
Hu I	19C11	42.4	(2) H28, 48	0
Hu II	1H11	53.4	(4) H9, 28, 91, L49	0
	9A3	43.9	(3) H9, 28, 91	0
	CW43	10.53	(3) H9, 28, 91	HCDR3, ^{101}A→R
	Y49K†	53.4	(4) H9, 28, 91, L49	HCDR3, ^{101}A→R
Hu III	2B12	4.67	(5) H9, 28, 38, 46, 48	HCDR3, ^{101}A→K
	2B12	4.67	(5) H9, 28, 38, 46, 48	HCDR3, ^{101}A→K
	2B8	2.81	(1) H28	HCDR3, ^{101}A→K; LCDR3, ^{96}R→Y
	F4	0.24	(1) H28	HCDR3, ^{101}A→K; LCDR3, ^{96}R→W
Hu IV	L3.17	0.10	(1) H28	HCDR3, ^{101}A→K LCDR3, ^{94}F→Y LCDR3, ^{96}R→W

*The number of murine framework residues that differ from the most homologous human germline sequence based on definition of CDRs of Kabat et. al. (1977, 1991) are indicated in parentheses. Differing murine framework residues retained in the humanized versions are located predominantly on the H chain (H) at the indicated positions. Hu I clone 1H11 and the CW43 derivative, clone Y49K, contain a single differing L chain (L) framework residue at position 49.

†Clone Y49K was created by site-directed mutagenesis of clone CW43. The four clones within the shaded boxed region, 1H11, 9A3, CW43, and Y49K, were characterized to

demonstrate the co-operative interaction between L chain framework residue tyr^{49} (human) and HCDR3 residue arg^{101}.

The variants displaying enhanced affinity were tested for their ability to block the binding of gp39 ligand to the CD40 receptor. Immulon II microtiter plates were coated with 2 μg/ml anti-murine CD8 to capture sgp39 fusion protein which expresses the CD8 domain. The plates were rinsed once with PBS containing 0.05% Tween 20, and were blocked with 3% BSA in PBS. The plate was washed once with PBS containing 0.05% Tween 20 and was incubated with cell culture media containing saturating levels of sgp39 for 2 h at 25°C. Unbound sgp39 was aspirated and the plate was washed two times with PBS containing 0.05% Tween 20. Next, 25 μl of purified variant Fabs diluted serially 3-fold in PBS was added followed by 25 μl of 4 μg/ml CD40-human Ig in PBS. The plates were incubated 2 h at 25°C and were washed three times with PBS containing 0.05% Tween 20. Bound CD40-human Ig was detected following a 1 h incubation at 25°C with goat $F(ab')_2$ anti-human IgG Fcγ-specific horseradish peroxidase conjugate (Jackson) diluted 10,000-fold in PBS. The plate was washed four times with PBS containing 0.05% Tween 20 and binding was quantitated colorimetrically by incubating with 1 mg/ml *o*-phenylenediamine dihydrochloride and 0.003% hydrogen peroxide in 50 mM citric acid, 100 mM Na_2HPO_4, pH 5. The reaction was terminated by the addition of H_2SO_4 to a final concentration of 0.36 M and the absorbance at 490 nm was determined. Figure 2B shows purified variants were tested for their ability to inhibit sgp39 binding to CD40-Ig. The ligand for the CD40 receptor, gp39, was captured in a microtiter plate and subsequently, varying amounts of purified chimeric (filled circles), Hu II-CW43 (open squares), Hu III-2B8 (filled triangles), Hu

II/III-2B12 (open triangles), and irrelevant (filled squares) Fab were co-incubated with 2 μg/ml CD40-human Ig on the microtiter plate. The variants all inhibited the binding of soluble CD40-Ig fusion protein to immobilized gp39 antigen in a dose-dependent manner that correlated with the affinity of the Fabs. For example, one of the most potent inhibitors of ligand binding to CD40-Ig fusion protein was variant 2B8, which was also one of the variants with the highest affinity for CD40. Variant 2B8 displayed ≈17-fold higher affinity for CD40 than did the chimeric Fab and inhibited ligand binding ≈7-fold more effectively.

Screening of the Hu I library identified two variants that were similar or identical in framework sequence to the Hu II clone CW43 but displayed 5-fold lower affinities (Table II, clones 1H11 and 9A3). Clone 9A3 has an identical framework structure while 1H11 contained the murine lysine framework residue at L chain position 49. Sequence comparisons and site-directed mutagenesis studies (data not shown) suggest that the beneficial arginine residue at HCDR3 position 101 might interact with L chain residue tyr^{49}. To test this, L chain residue tyr^{49} of clone CW43 was mutated to the lysine murine framework residue, resulting in a variant with a framework identical to clone 1H11 that also contained the beneficial arg^{101} residue in HCDR3. The resulting mAb, termed Y49K, displayed 5-fold lower affinity than CW43. Thus, expression of tyrosine at L chain framework residue 49 or expression of arginine at HCDR3 residue 101 alone had no beneficial effect on the mAb affinity, while the concomitant expression of tyrosine and arginine at these sites improved the mAb affinity 5-fold. The non-additive, or dependent nature of the mutations demonstrates that L chain residue tyr^{49}